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THE PREPARATION OF DIPHTHERIA ANTITOXINE.

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THE PREPARATION OF DIPHTHERIA ANTITOXINE.

THE general principles involved in the preparation of diphtheria antitoxine have recently become familiar to all medical men. Their recapitulation does not therefore seem essential for the purposes of the present paper, which are, first, to note the events of interest occurring during the prosecution of some recent work on the subject; and second, to direct attention to certain points of difficulty which have been encountered.

In view of the limited time at my disposal it appears preferable to omit detailed reference to the preparation of culture media and to the selection of special bacteriological apparatus. It will be sufficient to state that full accounts of the former are to be found in numerous recent works, while complete catalogues devoted solely to this subject have been issued by several manufacturers of scientific supplies.

The acquisition of a culture of diphtheria that will yield the requisite amount of toxine under specified conditions is a matter of the first importance. One culture may be a hundred times as productive of toxine as another, although both are grown under the same conditions. My own experience indicates that a satisfactory culture is something of a rarity. Cultures were tested from eight cases of severe clinical diphtheria in the following way: From each case a flask of bouillon was inoculated, and the virulence of the

fresh twenty-four hour culture tested by injecting into a guinea pig one cubic centimeter. From the two pigs which died first within two days, pure cultures of the diphtheria bacillus were again recovered, inoculated into bouillon, and grown under proper conditions for three weeks. At the end of that time the bouillon of one flask was found to be nearly twice as rich in toxine as the other, and this culture was used exclusively in the subsequent work. The virulence of this bacillus and its toxic productiveness have remained unimpaired after more than a hundred re-inoculations, covering a period of eight months.

When a flask of bouillon is inoculated with the diphtheria bacillus the following phenomena occur: At the end of a day or two the bouillon is generally turbid throughout, but with little or no sediment. Although the bacilli are now at the stage of maximum virulence, yet no appreciable amount of toxine is to be found, as may be shown by filtering off the bacilli, and injecting the clear filtrate into guinea pigs. Large doses of this produce often no more effect than an equal amount of ordinary bouillon. At the end of a week a distinct sediment settles, and a thin translucent layer of bacilli can be seen floating on the surface of the liquid. Microscopical examination shows what is taking place: The sediment consists of dead bacilli, disintegrated, and no longer identifiable, while the surface layer is composed of living, rapidly multiplying bacteria of characteristic appearance. Toxine can now be demonstrated in the fluid.

During the next few weeks the sediment increases, often forming more or less coherent flakes and lumps, the superficial layer becomes thicker, and more opaque, and is composed of scaly particles. If these be once separated by shaking they show no tendency to coalesce, but remain discrete while the intervening surface becomes covered with a film, slowly and progressively increasing in opacity. After

five or six weeks the surface layer often sinks and disappears; examination shows very few living bacilli, but the fluid is rich in toxine.

It has been stated that toxine is not secreted by the living bacilli, but is the product of their disintegration after death. Whether this be so or not, the amount of toxine in a given bouillon culture is proportionate to the amount of sediment, that is, to the number of bacilli which have completed their cycle in the liquid. Multiplication of the bacilli is favored by access of oxygen, and it has been consequently recommended to grow the bouillon cultures in a current of air. It is unquestionably true that a bouillon culture three inches deep yields proportionately less toxine at the end of a month than one one inch in depth, there being in the latter instance a proportionately greater air surface. While oxygen is thus shown to be required, probably but a small amount is made use of, and I have not found that bouillon cultures one-half inch deep, grown in a current of moist air, yield more toxine than when grown in the ordinary cotton plugged flask. Provided the layer of fluid be shallow the interchange of air through the cotton plug is probably sufficient for the needs of the bacteria. The risk of contamination is always greater even with the strictest precautions where there is a current of air entering the flasks: and, provided there is sufficient incubator space, the ordinary method seems to me preferable.

The toxic strength of each lot of toxic bouillon is to be carefully determined. If of standard strength, one-tenth of a cubic centimeter should prove fatal to a guinea pig in about thirty-six hours. The amount of toxic bouillon constituting the minimum fatal dose for a guinea pig under these circumstances is a toxine unit. Since in the course of this work the toxic strength of the bouillon employed has not been constant, it seems preferable to designate the doses injected into the horses, as toxine units, rather than so

many cubic centimeters of toxine. Thus at the outset one cubic centimeter of the bouillon contained five toxine units, and was consequently of half the standard strength, while later the standard strength of ten toxine units to the cubic centimeter was obtained.

For the filtration of the toxine from its detritus and still living bacilli, the Pasteur filter was used.*

The filtrate thus obtained from the bouillon is absolutely clear and sterile, but is, however, an excellent culture medium for bacteria that may find entrance. I have used as a preservative a ten per cent. solution of thymol in chloroform, which, added to the toxine in the proportion of one per cent., has efficiently checked the development of foreign germs.

With reference to choosing horses for the purpose of immunization, my experience is too limited to justify the formulation of definite rules. It is impossible to tell at the outset what horses will stand the process sufficiently well

* A large stout glass cylinder is constricted abruptly at one end to a diameter of an inch, and this drawn out to a tapering extremity. A Pasteur filter is fitted by a rubber stopper to the constriction within the cylinder, and the tapering end of this adjusted to a filtering flask. When now the cylinder is filled with liquid, and suction applied to the filtering flask, atmospheric pressure forces the liquid through the porous porcelain, and the filtrate falls drop by drop into the filtering flask. As the level of the liquid in the cylinder falls below the top of the bougie, less and less filtering surface is at work. To obviate this disadvantage a large test tube of such a diameter as to fit easily over the bougie is filled with the liquid, and by a sudden movement inverted over the bougie, its lower end resting upon the projecting shoulder of the bougie. The bougie is thus surrounded by a column of fluid which is sustained by atmospheric pressure.

The remainder of the fluid is now poured into the cylinder and air exhausted from the filtering flask. Filtration now proceeds with the whole of the filter actively at work, until the last few cubic centimeters of liquid in the cylinder are reached and the level sinks to the bottom of the test tube. Bubbles of air are then drawn into the test tube, the column in it falls, and the filtration is complete. The apparatus admits of ready sterilization in separate pieces, and is exceedingly easy to manipulate. The filtering flask may be connected by a Y fork with a glass tube inserted into a bottle of mercury, thus enabling one to judge, by the height of the column of mercury in the tube, of the amount of vacuum obtained. I have found an air pump more satisfactory than the water aspirator. By interposing a large stout bottle between the air pump and the filtering flask a single exhaust will produce a vacuum sufficient to filter a liter of toxine.

to be brought to a high degree of immunity, and what, on the other hand, will prove of only moderate value. A horse somewhat thin, with soft muscles, is easier to manage than a stout one, since the injections are more readily made, and the jugular vein easier of access. A horse of a vicious or nervous disposition is neither agreeable nor safe for the operator. The mallein test should be made before beginning treatment.

The process of immunizing the horses requires good judgment and extreme care. As to their feeding, it seems to me that until they have been bled they should not be treated very differently from any horse leading an inactive life. After each tapping a generous diet should be allowed. The weights and general condition are to be most carefully watched, and on any falling off the injections are to be suspended. As will be shown later this is a point of extreme importance.

For the injection of large amounts of toxine into the horses a syringe was used holding 100 c.c. The interposition of a short bit of rubber tubing between the needle and the syringe was found necessary to allow for sudden movements of the animal during the process. The short stout tuberculin needle of the veterinarians causes practically no pain on being introduced, if the animal's attention be distracted by the application of an implement known as a "twitch" held on the nose or ear by the assistant. The loose skin just back or in front of the foreshoulder are most suitable places for injection. It is also easy to introduce the toxine intravenously by compressing the external jugular vein, and thrusting the needle into the distended vessel.

The immunization of five horses for the Massachusetts State Board of Health was begun in the second week of December, 1894. During the first month of the inoculations the animals exhibited essentially similar and uneventful clinical histories. The initial dose of one half the nor-

mal toxine unit was gradually increased every other day until at the end of four weeks fifteen units were injected. Up to this time no reaction of any sort was apparent. This gradual commencement seems justified by clinical and experimental analogy. The sudden introduction of diphtheria poison into the organism of an animal totally unused to such toxic shocks may be productive of tissue changes apparent only after the lapse of considerable time. I have repeatedly observed that a minute dose of toxine in a guinea pig, scarcely sufficient to cause malaise, has been followed after two or three weeks by paralysis of the extremities, cachexia, and progressive emaciation. On these theoretical grounds, therefore, the attempt was made to avoid any marked reaction at the outset.

After the first month the animals began to show widely differing degrees of susceptibility. The clinical histories of two horses will illustrate respectively the extremes of tolerance and of sensitiveness.

A small mare of good appearance, with soft muscles, received fifteen toxine units at the end of the first month with absolutely no reaction. The doses were then rapidly increased twice a week till at the end of seven weeks she was receiving 500 units, at nine weeks 1500 units, with slight local reaction, and a week later a dose of 2500 units, with but slight local, and no constitutional symptoms. On March 5 a preliminary tapping was made, and the serum was found to possess an immunizing value of at least 1–50,000.

During the next two weeks 5,000 toxine units were introduced without ill effect, and on March 23 six liters of blood were withdrawn. The value of this serum was found to be not under 1–75,000. This strength had therefore been attained in a little over three months from the beginning of the inoculations.

In contrast with this rapid immunization, another horse

equally sound, but more irritable and difficult to handle, began to experience at the end of the first month after each dose of 15 units, local swelling lasting several days. At six weeks 100 units were followed by considerable swelling lasting a week. At the end of two months 500 units were followed by enormous swelling of the foreshoulder, with general œdema of the whole extremity. In a week this had gone, and a repetition of the dose was followed by much less reaction. During the third month injections of 1000 units were followed by very hard indurations about eight inches in diameter, each requiring for subsidence about ten days. The injection of 3000 units at the end of four and a half months was followed by enormous reaction, a rock-like induration eighteen inches across, with sharply defined edges, showing no tendency to central softening or suppuration, disappearing after three weeks irregularly, leaving several discrete masses which finally vanished. Then 4000 units excited but moderate reaction, which disappeared in a week.

The horse was tapped at the end of five months and a half, and showed a serum of the strength of 1–75,000.

Between these two extremes the intermediate spaces could be filled in by the histories of the other animals. A detailed account would involve much repetition, and it seems preferable to state in the form of a summary the phenomena which have been observed.

In the first instance the local reaction appears to stand in direct relation to the susceptibility of the animal at the moment of injection. In a sensitive animal an injection of toxine is followed by a pronounced reaction lasting from a few hours to several weeks. If the injection is made into the subcutaneous tissue the tumefaction is soft and of the nature of an œdema, while if made into the muscle it is firm, at times of rocky hardness. This swelling may remain small, being not more than two or three inches in diameter,

or it may increase in size, attaining a diameter of two feet or more, extending beyond the shoulder and even travelling along the trunk as a well-defined œdematous area. A reaction of this extent is generally sufficient cause for grave anxiety. These extensive reactions are especially apt to occur after an animal has been bled and his powers of resistance consequently diminished. It is common for a horse which has shown no especial sensitiveness during the process of immunizing to manifest, after tapping, a marked reaction from the injection of one half or one quarter of the amount previously given without ill effect. Furthermore a horse which has begun to lose weight may, if the injections are continued, unexpectedly develop one of these pronounced and serious disturbances. These local reactions may be complicated by the entrance of foreign bacteria. Abscesses have occasionally formed and healed after evacuation without untoward incident. A more serious event occurred in a horse which had been immunized rapidly and easily. After the second tapping an injection of 2500 toxine units was followed by enormous firm swelling of the foreshoulder œdema of the extremity and neighboring portion of the trunk, death supervening in four days without characteristic symptoms. Incision into the tumefaction at the shoulder showed at the depth of an inch below the skin a collection of reddish serous fluid and large quantities of foul smelling gas. Undoubtedly some foreign organism had gained entrance and found a suitable field for development in the exudation consequent upon the injection, but from lack of facilities for anærobic cultivation its nature was not determined.

Constitutional disturbances have been much less pronounced than might be expected. Even with the most severe local reactions no general symptoms could be perceived beyond a loss of appetite and spirits. In one instance, however, the intravenous injection of toxine was followed by pronounced effect. A horse that received without ill effect

3000 units subcutaneously, exhibited on the introduction of an equal amount into the jugular vein, in the course of half an hour, sweating, trembling, faintness, and loss of appetite. The symptoms passed off in a few hours, and the horse appeared as usual by the following morning.

This procedure appeared to be attended with some risk and was not repeated.

The process of tapping is comparatively simple, and may be completed in fifteen minutes. One or two assistants, skilful in the management of horses, are needed. The animal submits most readily to the operation if not confronted with elaborate preparations for securing him. He is backed into a short narrow stall, so arranged that his neck protrudes from it, and he is unable to move sideways or backwards. The head is firmly held, a rope is passed around the neck low down and a wedge thrust beneath the rope so as to compress the external jugular vein, which is immediately seen to stand out for several inches below the angle of the jaw. A syringe full of two per cent. cocaine is injected over the vessel where it appears most superficial, and a short incision made, exposing it. It is generally recommended to thrust a trocar into the vein and conduct the blood into a jar through a rubber tube. I have found it more simple and expeditious to incise the vein directly: the blood then spurts in a steady stream for several feet. The receiving jar is placed close to the incision, and the blood allowed to flow into it without splashing, which is apt to alarm the horse. When four to eight liters are collected, the tourniquet is withdrawn, and the flowing generally ceases. To insure safety a pin is thrust through the skin flaps, which are then brought together by a thread in figure of 8 loops. The horse is not allowed his head for a day, but is fed from a rack to avoid the danger of hemorrhage.

The amount of serum that separates is dependent upon the completeness of the contraction of the fibrin in the clot.

If the clot adheres to the sides of the jar, contraction of the fibrin is prevented, and at the end of several days the clot is still firm, and but a few drops of serum have separated. If, however, the clot is separated from the sides of the jar, contraction immediately begins with a simultaneous forcing out of serum.

The one factor that more than any other promotes contraction of the clot, is the presence of a large surface in proportion to the volume of blood. Thus a jar, half filled, yields proportionately more serum than one entirely filled. The same point is shown by the fact that a partially filled jar, in which the blood has coagulated at a sharp incline, yields more serum than the same amount of blood coagulated in an upright jar.

After many experiments the following method was found to yield the most satisfactory results. Cylindrical jars of one liter capacity are used, and vaseline is thoroughly applied to the interior to prevent adhesion of the clot to the sides. A large test tube filled with cracked ice, closed at the top by a perforated rubber stopper, is placed in each jar before receiving the blood. When filled the jar is placed on ice, and the blood slowly coagulates, becoming a firm mass in the course of several hours. The test tube is then withdrawn from each jar, leaving a cylindrical deficiency in the clot. At the end of twenty-four hours the result is striking. The clot has shrunken to a soft, flabby mass, and is surrounded by abundance of clear yellow serum. This is removed by carefully pouring off, and constitutes about two-thirds of the total amount obtainable. If the jars are placed on the ice another twenty-four hours, about one-third more may be obtained. This remainder is colored by solution of pigment in varying degrees, but is equal in all therapeutic qualities to the first yellow serum. The strength is ascertained by either the French or German method. The former was adopted at the beginning of the work, but

I have recently come to regard the latter as more scientific and accurate.

The serum is next passed through a Pasteur filter to ensure sterility, and the filtrate shaken with the chloroform-thymol solution previously mentioned. It is, however, not allowed to stand long in contact with an excess of the solution, since a precipitate readily forms, but is immediately transferred to sterilized bottles, and aseptically sealed, being then ready for use.